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TITLE: The Role of Telomeric Repeat Binding Factor 1 (TRF1) in Telomere Maintenance and as a Potential Prognostic Indicator in Human Breast Cancer

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13. ABSTRACT (Maximum 200 Words)
The aims of this study are to (i) determine the relationships between the telomere binding protein Telomere Repeat Binding Factor 1 (TRF1) and other telomere binding proteins, (ii) establish the potential of TRF1 as a surrogate marker for telomere content (TC) and as a potential clinical marker and (iii) characterize the relationship between of the telomere binding protein TRF1 and TC. Through examining the role of TRF1 in telomere length control and in breast cancer progression, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are on schedule. The research is in progress.

14. SUBJECT TERMS
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I. INTRODUCTION

The aims of this study are to (i) determine the relationships between the telomere binding protein Telomere Repeat Binding Factor 1 (TRF1) and other telomere binding proteins, (ii) establish the potential of TRF1 as a surrogate marker for telomere content (TC) and as a potential clinical marker and (iii) characterize the relationship between of the telomere binding protein TRF1 and TC. Through examining the role of TRF1 in telomere length control and in breast cancer progression, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work are on schedule. The research is in progress.

Hypothesis and Rationale

Studies from the Griffith Lab demonstrate that Telomere Content (TC) has prognostic value in breast and prostate cancer; however the factors that control TC are poorly understood. In vitro studies have shown that high levels of TRF1 can stabilize short telomeres and the preliminary results suggest the levels of TRF1 mRNA are related to TC. Together this data suggests that TRF1 level may be directly related to telomere content and therefore be a potential biomarker for telomere length. However, TRF1 also has multiple interacting partners, TRF1 Interacting Nuclear Factor 2 (TIN2), Tankyrase, Telomere Repeat Binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which may modify the interaction between TRF1 and TC. If increases in TRF1 are partially responsible for decreased TC, a prognostic marker of poor outcome, then targeting TRF1 may be a good preventive treatment of breast cancer progression. However, it is also possible that the observed increase in TRF1 is a cellular reaction in response to low TC and therefore a good surrogate for TC. Theses two scenarios must be tested to evaluate the prognostic significance of TRF1 in human breast cancer. Therefore *I hypothesize that defining TRF1 levels using immunohistochemistry could provide a surrogate measure for TC that would be easily adaptable to the clinical setting.* To test this hypothesis, I will assess the potential prognostic value of the TRF1 in human breast tumor samples. Additionally, I propose to characterize the relationship of TRF1 to TC, and to TIN2 and Tankyrase to further examine the relationship of TRF1 to TC. I will evaluate this hypothesis through three specific aims.

- **Specific Aim #1**
  Assess the potential of TRF1 protein levels as a surrogate for Telomere DNA Content (TC) in frozen and paraffin embedded breast tumor tissues.

- **Specific Aim #2**
  Assess the potential modification of the relationship between TC and TRF1 mRNA levels by TRF1 interacting protein 2 (TIN2) and Tankyrase in frozen human breast tumor samples.

- **Specific Aim #3**
  Examine the effects of increased TRF1 expression on TC and decreased TC on TRF1 expression in breast cancer cell lines.

II. KEY RESEARCH ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

I have demonstrated the following:

- An association between the levels of TRF1, TIN2 and POT1 mRNA within breast tumors, as measured by real-time RT-PCR (Appendix A).
- The levels of TRF1mRNA are not associated with the mRNA levels of the human telomerase reverse transcriptase (hTERT) mRNA or the levels of TRF2 mRNA within breast tumors (Appendix A).
- The levels of TIN2, TRF1, TRF2 and POT1 mRNA are all associated with telomere content (Appendix B).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of the award, the PhD candidate has had the opportunity to work and interact with oncologists, pathologists and other PhD scientists who specialize in breast cancer. These interactions have occurred though tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. The candidate has been trained by experts that oversee the Cancer Center Fluorescence Microscopy Facility at the UNM Cancer Research Facility.

On an educational level, the candidate has been co-instructing two upper-level undergraduate courses, Biochemical Laboratory Methods and Intensive Biochemistry II: Intermediary Metabolism. The candidate has also been mentoring undergraduate students in laboratory techniques and presentation preparation. The candidate has aspirations of continuing her career in research and remaining in academia and felt teaching provided an opportunity to develop the essential teaching skills need for her chosen career.

IIc. PERFORMANCE ACCOMPLISHMENTS:

Experimental Milestones

Specific Aim 1: (7 tasks)

Task 1 Month 1-2 **Complete**

- Purify DNA from paraffin embedded breast tumor samples previously collected by our laboratory.

Task 2 Month 2-6 **Complete**

- Measure TC in paraffin embedded breast tumors samples.

Task 3 Month 6-12 **Complete**

- Optimize TRF1 antibody for use in frozen tissue and paraffin embedded breast tumor tissue.

  TRF1 antibody specificity has been demonstrated in breast cancer cell-line MCF-7 and conditions for antigen retrieval and staining have been determined.

Task 4 Month 12-14 **Not Initiated**

- Section frozen human breast tumor samples and stain with antibody to TRF1.

Task 5 Month 13-14 **Not Initiated**

- Assess relationship between normalized TRF1 mRNA levels and TRF1 staining intensity.

Task 6 Months 14-24 **Not Initiated**

- Section paraffin embedded breast tumor tissues and stain with antibody to TRF1.

Task 7 Months 12-30 **Not Initiated**

- Score sections stained with TRF1 antibody and compare to TC data, histological markers and survival data.

Specific Aim 2: (4 tasks)

Task 1 Month 1-2 **Completed**

- Extract RNA from frozen breast tumor samples already collected by our laboratory. Design and order Tankyrase and TIN2 primers and probe.

  RNA was extracted from 36 breast tumors. Primers for Tankyrase and TIN2 were designed.

Task 2 Month 2-4 **Partially Successful**

- Optimize Tankyrase and TIN2 RT-PCR

TIN2 RT-PCR was optimized, however Tankyrase primers detected both Tankyrase 1 and the analog Tankyrase 2. The expression levels of these two proteins are quite different and Tankyrase 2 is highly expressed and functionally not associated with telomere length control. Assessment of Tankyrase by RT-PCR yielded an inconclusive result in all experiments. RT-PCR experiments to determine Tankyrase mRNA levels have been deferred pending new methods to delineate these two analogs at the molecular level. Recent studies have determined the regulation of telomere length by proteins to involve a number of complexes, which include TRF1 and TIN2, and also include Tankyrase, POT1 and TRF2. As levels of Tankyrase mRNA could not be determined, and POT1 and TRF2 levels may be associated with TRF1 mRNA levels and involved in telomere content determination, RT-PCR reactions were optimized for TRF2 and POT1 as well.

Task 3 Month 4-7 **Completed**

- Measure Tankyrase and TIN2 mRNA levels by RT-PCR in RNA extracted from frozen breast cancer samples.

Tankyrase mRNA levels could not be assessed, however TIN2, POT1 and TRF2 mRNA levels were assessed in 36 frozen breast tumor samples.

Task 4 Month 7-12 **Completed**

- Analyze association between Tankyrase and TIN2 mRNA levels with TC and TRF1 mRNA expression.

Tankyrase mRNA levels could not be assessed so no comparison was possible. TIN2 mRNA levels showed a strong association with TC and TRF1 levels as well as two other telomere binding proteins; POT1 and TRF2 (Appendix A-C).

Specific Aim 3: (6 tasks)

Task 1 Month 12-15 **Not Initiated**

- Design and test small interfering RNAs (siRNA) of human Telomerase Reverse Transcriptase (hTERT)

Task 2 Month 15-27 **Not Initiated**

- Express siRNA of hTERT in breast cancer cell lines and examine TRF1 mRNA levels and TC by RT-PCR and slot blot over time.

Task 3 Month 15-18 **Not Initiated**

- Design, generate and test TRF1 expression vector.

Task 4 Month 18-30 **Not Initiated**

- Overexpress TRF1 in breast cancer cell lines and examine TC levels by slot blot over time.

Task 5 Month 30-34 **Not Initiated**

- Analyze relationship between TRF1 and TC.

Task 6 Months 30-36 **In Progress**

- Prepare and submit manuscripts.

A paper detailing the results of Specific Aim 2 has been submitted and is currently in review.

Education and Training Milestones (6 tasks)

Task 1 Month 1-6 **Complete**

- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste.

Student has examined various types of breast cancer and can recognize features of different tumors and tumor stages.

Task 2 Month 1-36 **In Progress**

- Attend tumor board meetings and monthly Cancer Research and Treatment Center Meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.

Task 3 Month 1-6 **Delayed**

- Attend the University of New Mexico School of Medicine medical student training Neoplasia block.

Student postponed attending the Neoplasia block, due to scheduling conflict with required PhD coursework. Student will attend the Neoplasia block this coming fall.

Task 4 Month 6-12 **Completed**

- Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste.

Student has learned basic staining procedures and has gained understanding of the commonly used markers to determine treatment in breast cancer.

Task 5 Month 12-24 **In Progress**

- Work with oncologists in the University of New Mexico Hospital to gain perspective on breast cancer.

Student has initiated contact and been meeting with oncologists to understand the process of breast cancer diagnosis, treatment and management.

Task 6 Months 12-36 **In Progress**

- Present ongoing work at local and national meetings

III. REPORTABLE OUTCOMES

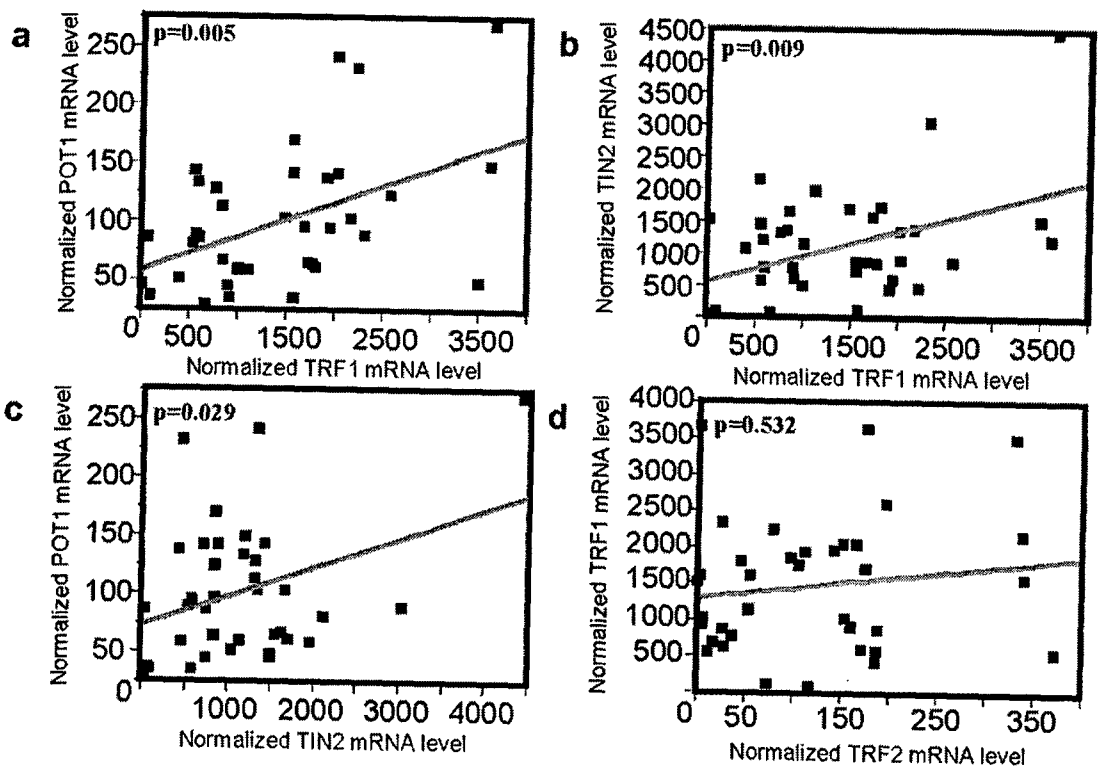
Presentations: (abstract in Appendix)

AACR Special Conference: "The Roles of Telomeres and Telomerase in Cancer" San Francisco, Nov 3-7, 2004. "Levels of Telomere Protein mRNAs are Predictive of Telomere Content in Human Breast Tumors." Kimberly S. Butler, William C. Hines, Diana Roberts, Colleen A. Fordyce, Jeffrey K Griffith (Appendix C).

IV. CONCLUSIONS

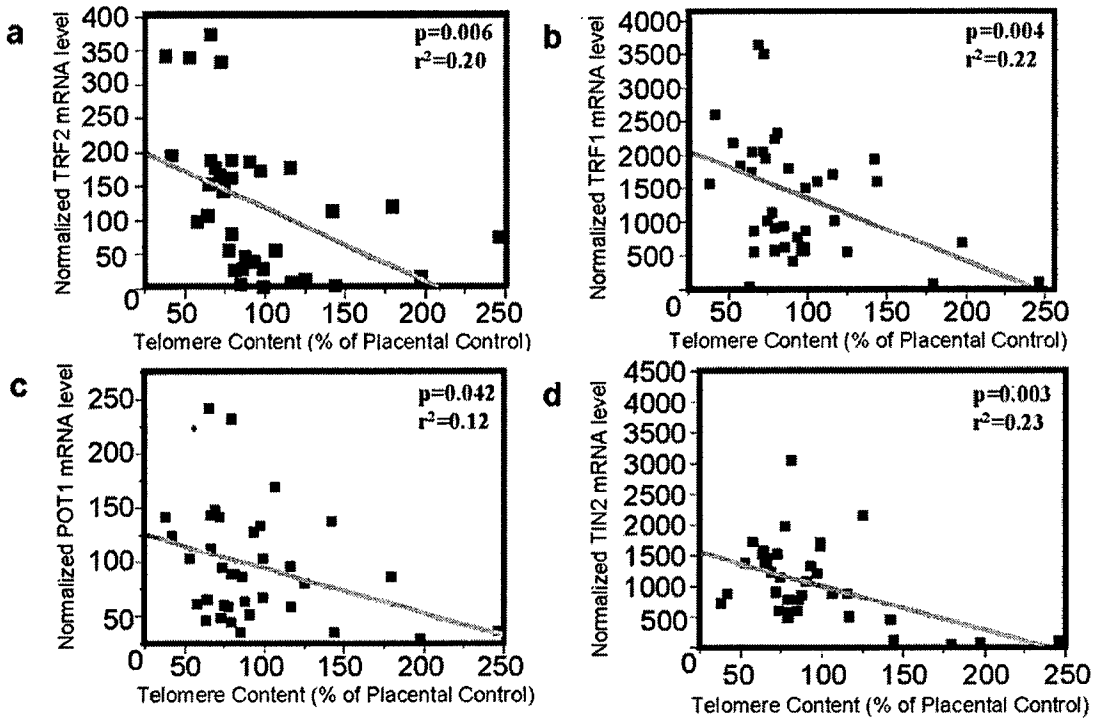
To date, all tasks; as outlined in the Statement of Work are on schedule. The tasks contained in Specific Aim #1 have been initiated and are proceeding on schedule. The immunohistochemical assay for TRF1 has been optimized and work in tissue should begin shortly. Specific Aim #2 has been completed and a manuscript based on the results from this body of work has been submitted for review. The tasks for Specific Aim #3 have not yet been initiated, but work on these tasks is planned to follow the schedule laid forth in the Statement of Work. The Ph.D. candidate has met all but one of her educational goals and is expecting to attend the neoplasia block at the University of New Mexico School of Medicine the next semester.

Appendix A



Relationship between levels of mRNAs for telomere associated proteins.
The mRNA levels of TRF1, TRF2, POT1 and TIN2 were assessed by quantitative RT-PCR in 36 human breast tumors. **A.** POT1 mRNA levels were compared to TRF1 mRNA levels, showing a significant positive association (p=0.005). **B.** TIN2 mRNA levels were compared to TRF1 mRNA levels, showing a significant positive association (p=0.009). **C.** POT1 mRNA levels were compared to TIN2 mRNA levels, showing a significant positive association (p=0.029). **D.** TRF1 mRNA levels were compared to TRF2 mRNA levels, showing a representative graph of the comparison of TRF2 mRNA levels to any of the other telomere associated proteins mRNA levels, to which no association was noted (p=0.532). No associations between hTERT and TRF1, TRF2, TIN2 or POT1 were found.

Appendix B



Relationships between Levels of mRNAs for Telomere associated proteins and Telomere DNA Content (TC).

TC was compared to mRNA levels of TRF1, TRF2, POT1 and TIN2 levels assessed by quantitative RT-PCR in 36 human breast tumors. **A.** TRF2 mRNA level compared to TC, this graph shows the trend for increased TRF1 mRNA levels with decreased TC (p=0.006). **B.** TRF1 mRNA level compared to TC, this graph shows the trend for increased TRF1 mRNA levels with decreased TC (p=0.004). **C.** POT1 mRNA level compared to TC, this graph shows the trend for increased TRF1 mRNA levels with decreased TC (p=0.042). **D.** TIN2 mRNA level compared to TC, this graph shows the trend for increased TRF1 mRNA levels with decreased TC (p=0.003).

Appendix C

Levels of Telomere Protein mRNAs are Predictive of Telomere Content in Human Breast Tumors

Kimberly S. Butler BA[1], William C. Hines BS[1], Diana Roberts MS[2], Colleen A. Fordyce[1] PhD, and Jeffrey K. Griffith PhD[1].

[1]Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico

[2]Department of Mathematics and Statistics, University of New Mexico, Albuquerque, New Mexico

The mechanism by which telomere length is maintained in human tissues is poorly understood. A model of telomere length control has emerged from previous studies in human cell lines, in which telomere-associated proteins act through two separate mechanisms to control telomere length. In the first mechanism, levels of TRF2 determine the rate of telomere attrition and the minimal telomere length in non-senescent human cells. In the second mechanism, the TRF1 complex controls the loading of POT1 onto the telomere, which prevents telomerase from elongating the telomere. Based on this complex model, we hypothesize that the relative levels of several or all of these telomere-binding proteins are determinants of telomere length in human tumors.

In the present study, we assessed the relationships between the mRNA levels of TRF1, TRF2, TIN2, POT1 and the telomerase protein component (TERT) to telomere length in 36 human breast tumors. Telomere length was measured as telomere content by the Telomere DNA Content Assay. Levels of the telomere-associated proteins mRNA were assessed by quantitative RT-PCR. Statistical modeling was performed using SAS software version 8.2 and JMP 5.1.

Linear regression revealed significant, negative, linear associations between TC and the mRNA levels of TRF1, TRF2, TIN2 and POT1 ($p=0.004$; 0.006; 0.003 and 0.042). No significant association between TERT mRNA levels and TC was noted. The significant p value and weak r^2 for each of the individual mRNAs suggest no single factor controls telomere length. Therefore, the General Linear Model regression procedure was used for predicting TC using mRNA levels of the telomere-associated proteins. Using the partial F test, it was determined the mRNA levels of the telomere-associated proteins are all necessary to predict TC ($p<0.0001$). Next, all possible two-way interactions were tested. The results indicate that the two-way interactions are significant ($p=0.0026$, $r^2=0.76$). Subsequent analyses indicate that three-way interactions are also significant ($p=0.0690$, $r^2=0.89$). The increase in r^2 resulting from the inclusion of the three way interactions is statistically significant, supporting the conclusion that the three way interactions are necessary to predict TC.

To determine which specific interactions are significant in predicting TC, the three-way interaction with the least significant p-value was excluded and the regression model was repeated until only the interactions that were statistically significant at level $p=0.05$ remained. These data demonstrate that TRF1 and TRF2 are not independently

associated with TC, nor is the interaction between TRF1 and TRF2. However, the individual interactions of TRF1 with TIN2, POT1 and TERT (p=0.0103; 0.0129 and 0.0209) and TRF2 with TIN2, POT1 and TERT (p=0.0092; 0.0248 and0.0119) are required to predict TC. The separate interactions between TERT and TIN2 and POT1 (p=0.0268 and 0.0350) are also needed to predict TC. The data demonstrate all of the mRNA levels are required to predict TC in human breast tumors.

In summary, the present study provides further support for the complex model of telomere length regulation, which was derived from well-characterized human cell lines and suggests that this model is applicable to human breast tumors.